FRUITING IN *DICHOMITUS SQUALENS* (KARST) REID

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ABSTRACT - *Dichomitus squalens* (Karst) Reid (Syn.: *Polyporus anceps* Peck) produces fruiting bodies on malt extract agar or on wood blocks at 28°C in controlled conditions of humidity and light. The life cycle of this fungus is of the haploid dicaryotic type with a bifactorial (tetrapolar) interfertility system. Meiosis takes place according to the classical schemes in homobasidiomycetes resulting in the formation of four basidiospores per basidia. Basidiospores are predominantly uninucleate and germinate to produce uninucleate mycelium which is unable to fruit. Compatible pairings result in dicaryons with clamp-connections at every septum.

RÉSUMÉ - *Dichomitus squalens* (Karst) Reid (Syn.: *Polyporus anceps* Peck) fructifie en milieu gélosé à l'extrait de malt en boîte de Pétri ou sur éprouvettes de bois en conditions stériles à 28°C dans des conditions contrôlées d'humidité et de lumière. Le cycle de ce champignon est du type haploïde/dicaryotique avec un système d'interfertilité bifactoriel (tétrapolaire). La méiose a lieu conformément aux schémas classiquement observés chez les homobasidiomycètes avec formation de quatre basidiospores par baside. Les basidiospores sont majoritairement uninucléées et germent pour former un mycélium uninucléé incapable de fructifier. Les confrontations compatibles produisent des dicaryons comportant des anses d'anastomose à chaque article.

KEY WORDS : *Dichomitus squalens* - interfertility system - fruiting.

INTRODUCTION

Dichomitus squalens (Karst) Reid (Syn = *Polyporus anceps* Peck) belongs to Homobasidiomycetes, Polyporaceae. This fungus has been studied for its ability to degrade lignin in wood (Chang et al., 1980; Blanchette et al., 1988) and in straws (Zadrazil & Brunnert, 1982). Among the white rot fungi, this species degrades lignin relatively fast with somehow limited degradation of cellulose and hemicellulose when grown in suitable conditions (Agosin & Odier, 1985). *D. squalens* produces a laccase presumably involved

in lignin degradation (Petrovski et al., 1980). No lignin peroxidase has been detected in this species (Agosin, unpublished). Several extracellular enzymes involved in the degradation of cellulose and xylans by this fungus have been purified and characterised; they include endoglucanases (Rouau & Foglietti, 1985), cellobiohydrolases (Rouau & Odier, 1986), xylanases, α-L-arabinofuranosidase (Brillouet & Moulin, 1985) and the pattern of xylan degradation has been characterised (Agosin et al., 1988).

The first description of this species was by Peck in 1895 who observed fruiting bodies growing on stump of *Tsuga canadensis* Carr. In 1929, Mounce reported the mating system of *D. squalens* to be unifactorial. In 1939, Baxter & Manis defined more precisely the taxonomic position of *Polyporus anceps* compared to *Polyporus ellisianus* (Murr.) Sacc. and Trott. Fruiting was described by Long & Harsch (1918), Baxter & Manis (1939), Badcock (1944) and Nobles (1948). Nobles et al. (1957) reported that the mating system in *P. anceps* is bifactorial. This species was renamed *Trametes squalens* then *D. squalens* in relation with the high degree of branching of the hyphae.

Basic research concerning *D. squalens* requires better information concerning its life cycle and its interfertility system. Accurate determination of fruiting conditions is necessary for the development of a laboratory method for fruiting in sterile conditions. In this study the life cycle of *D. squalens* is characterised and different conditions allowing fruiting are evaluated for their possible use in classical genetics in this species.

MATERIALS AND METHODS

1. Microbial strain

D. squalens (Karst) Reid (Syn.: *Polyporus anceps* Peck) was obtained from the Centraalbureau Voor Schimmelcultures (Baarn, Netherlands) (CBS 432-34).

2. Cultivation methods

Culture media include: 1/ Liquid medium with malt extract 2 % (w/v) (unless stated otherwise) with 0.2% (w/v) yeast extract, pH 5 (designated liquid malt extract); 2/ Malt extract agar 1.5% containing 3% malt extract. Media for fruiting include in addition to malt extract agar: 1. malt extract (5%) agar (1.5%) on the surface of which is deposited ■ wood block (*Pinus* sp.) (Lucas & Fougerousse, 1982); 2. A wood meal supplemented medium (*Picea sitchensis* (Bong) Carr.) as described by Holt et al. (1983).

All media containing malt extract were sterilised by autoclaving for 20 min at 120°C. The wood meal medium was autoclaved 25 min at 120°C. Wood blocks (10 x 2.5 x 2.5 cm) were streaked then autoclaved for 40 min at 120°C.

Media for fruiting experiments were inoculated with a mycelium freshly grown in liquid malt extract (not longer than 1 week at 34°C). Cultures were incubated at 34°C in the dark until colonisation was complete.

Cultures on microscope slides were run as follows: a drop of liquid malt extract was deposited aseptically on a sterile slide, then inoculated with a small amount of mycelium. Incubation took place in a humid sterile chamber at 28°C for 65-68 h. Mycelium was stained by Giemsa (see below).

The three basic procedures for fruiting using malt extract agar, wood blocks and the wood meal media were as follows: malt extract agar: the malt extract agar medium was poured (30 ml) in Petri dishes (9 cm diameter) and inoculated with a mycelium fragment in the centre. Light when used was obtained with Sylvania Grolux lamps (210-270 lux at the level of cultures). Incubation was at 18-24°C (laboratory temperature). Humidity was maintained by addition of liquid malt extract at regular intervals. Wood blocks: the method used was according to Lucas & Fougerousse (1982), 250 ml of 5 % malt extract 1.5 % agar media was poored in 1 litre flasks. After inoculation and colonisation of the whole surface of the agar (1 week), a wood block was deposited onto the agar and incubation proceeded for another 15 days after which mycelium had colonised the wood block. Woods blocks were removed and mycelium on the surface was scrapped off. Wood blocks were introduced into a wood box (90 cm x 50 cm x 40 cm, 40 blocks per box) containing vermiculite. The wood box was covered by a transparent plastic film. Incubation was at ambient temperature with light (9 h light and 15 h darkness) or darkness as specified. Moisture was maintained by addition of water to the vermiculite. Boxes were opened 30 min for aeration every day. Wood meal (*Picea sitchensis*): the wood meal medium (medium 1 according to Holt et al., 1983) was distributed in 250 ml conical flasks (50 ml per flask). After inoculation and colonisation of the culture (around 15 days), water was added to compensate for evaporation. Incubation proceeded at ambient temperature as specified: either with light (9h / 15 h) or with light (12 h / 12 h) or in darkness. Cultures were aerated every week with a stream of water-saturated sterile air and 2 ml water was added in order to compensate for evaporation.

Lyophilisation of basidiospores was done in milk with 10 % inositol (w/v) as a protective agent.

3. Interfertility experiments

Mono-basidiospore isolates were obtained by allowing basidiospores to germinate on malt extract agar at 16°C for 24h. Germlings were picked with a steel nib and transferred on malt extract agar. After 2-3 weeks incubation, the absence of clamp-connections in hyphae was verified by microscopic observation. Pairings were subcultured on malt extract agar and incubation proceeded at 34°C during 2-4 weeks. Any formation of dicaryons with clamp-connections was observed under the microscope.

4. Cytological observations

Routine microscopic observations were in Congo red (1 g Congo red; 100 ml concentrated ammonia, Kühner, 1938). Nuclei were stained according to Giemsa using the following procedure adapted from Kühner (1949). Mycelium was grown on a microscope slide as described above. It was fixed

in absolute ethanol (20 min), rinsed with water (20 min), hydrolysed with HCl 1 N for 10 min and rinsed in running water (10 min) followed by successive immersion in absolute ethanol (60 min), ethyl ether (5 min), absolute ethanol (5 min) then rinsed in water (5 min), Giemsa R solution (35 drops Giemsa R, 30 ml distilled water). For staining of basidiospores, basidiospores suspensions were spread on a microscope slide, then dried. The Giemsa stain procedure was applied starting with hydrolysis with HCl.

Hymenium was stained according to Lu (1962) as modified by Zickler (1973).

Table 1: Main characteristics of Fruiting in *Dichomitus squalens*[1].
Tableau 1: Principales caractéristiques des fructifications de *Dichomitus squalens*.

Fruiting characteristics	Fruiting method[2]		
	malt extract agar	Wood block	Wood meal
delay (weeks) before fruiting[3]	3.5 ± 0.5	11.5 ± 2.5	11 ± 4
nb fruiting cult./ total nb of cult.	30/45	6/40	49/50
fruiting bodies per cult.	4.5 ± 0.5	1.5 ± 0.5	1.5 ± 0.5
fruiting body diam.	1.5 ± 0.5	3 ± 0.5	1.5 ± 0.5
nb basidiospores per cult. and per 24 h	$1.5\ 10^7 \pm 0.5$	$1.6\ 10^6 \pm 1.4$	$7.5\ 10^5 \pm 0.25$

1: data are mean and standard deviation.
2: culture conditions are as described in Materials and methods; photoperiod is 9h light and 15h darkness.
3: delay for fruiting is defined as the time between inoculation and formation of fruiting bodies with visible pores.

RESULTS

1. Fruiting conditions experiments

Fruiting was examined in mycelia grown on malt extract agar, wood blocks and wood meal. All three conditions allowed fruiting in suitable conditions of humidity and light (see below).

The primordium appears as white thick callus. Adult fruiting bodies show pores visible to the naked eye more or less regularly on the surface. Characteristics of the fruiting bodies are shown in table 1. The frequency of cultures giving rise to fruiting bodies was variable according to the methods, the wood block method being less efficient than malt extract agar and the wood meal method. The malt extract agar method was the fastest (3.5 weeks) and resulted in the formation of significantly more basidiospores than the two other methods.

The effects of culture medium composition and light on fruiting were investigated in experiments using the three basic fruiting procedures.

No fruiting bodies developed in absence of light in any fruiting method (Tab. 2). The characteristics of fruiting bodies (as described in tab. 1) were the same whether the photoperiod was 9 h or 12 h light.

Table 2: Effect of light on fruiting in *Dichotomus squalens*.
Tableau 2: Effet de la lumière sur la fructification chez *Dichomitus squalens*.

	Fruiting bodies formation		
Fruiting method	darkness	9h light	12h light
malt extract agar	0	+	ND
Wood blocks	0	+	ND
Wood meal	0	+	+

0 = vegetative mycelium, + = formation of fruiting body with visible pores, ND = not determined.

2. Life cycle, cytological observations, interfertility system

The dicaryotic strain CBS 432-34 showed cells of 15 μm and 35 μm in length and 0.9 μm and 1.3 μm in diameter. The two nuclei were at an equal distance from the septa and close to each other (Fig. 2) with the exception of apex cells in which nuclei were near the apex. Binucleate chlamydospores were also observed (Fig. 11). Branching of mycelium was observed with anastomosis (Fig. 3). In the hymenium, apical cells differentiated into binucleated basidia. Caryogamy was immediately followed by meiosis (Fig. 4,5,6). At the end of the second meiotic division, the number of nuclei was four and the extremity of basidia developed sterigmata (Fig. 7). Haploid nuclei migrated toward the four basidiospores formed at the extremity of sterigmata (Fig. 8,9). The size of basidia was 6-8 μm while basidiospores were 2-3 μm x 7-10 μm. Giemsa staining established that 90% of basidiospores were mononucleate, the rest being binucleate or empty (Fig. 10).

Twenty monobasidiospore isolates were examined by Giemsa staining (Fig. 1): all isolates were uninucleate and showed septa with a septal pore (dolipore). No clamps were observed in monobasidiospore isolates (homocaryons). Certain hyphal cells swelled considerably in comparison with adjacent cells, the protoplast contracted and a thick cell wall developed resulting in the formation of an asexual spore (chlamydospore).

Monobasidiospore isolates were combined with one another in all possible combinations. The formation of dicaryons showing clamp connections was examined in crossings. Results (Tab. 3) establish the existence of four classes of homocaryons in the progeny of the original dicaryon. Formation of dicaryons showing clamp connections showed no deviation from a tetrapolar mating system: a given homocaryon was able to form a dicaryon with clamp connections with all homocaryons of the compatible class and with no homocaryon of non compatible classes. However not all dicaryons were able to form fruiting bodies able to generate viable basidiospores (data not shown).

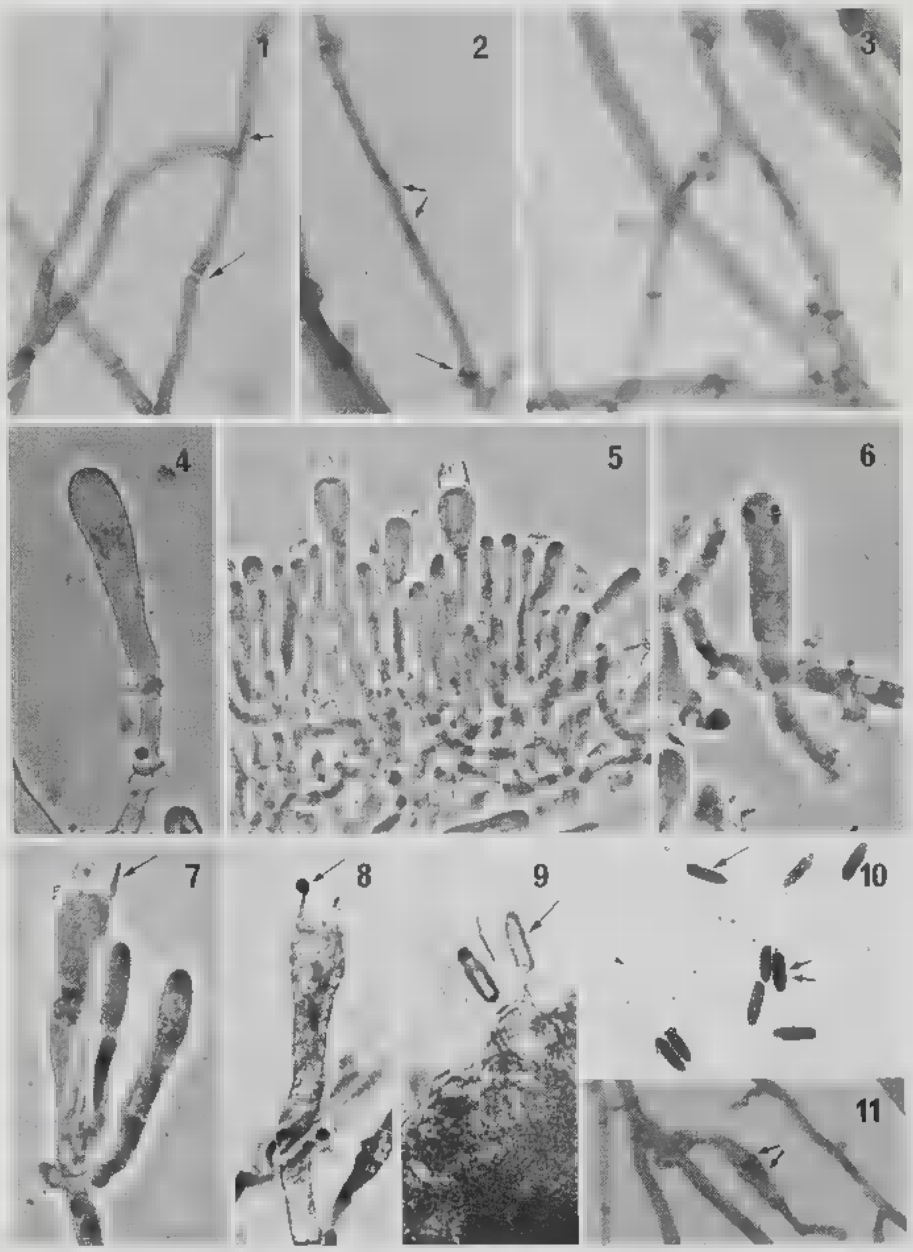

Table 3: Results of crossing experiments with homocaryons of *Dichomitus squalens*. +: presence of clamp connections, - : absence of clamp connections.
Tableau 3: Résultat des croisements entre homocaryons de *Dichomitus squalens*. +: présence d'anses d'anastomose, - : absence d'anses d'anastomose.

Dichotomus squalens at different states of its cycle: 1. Uninucleate homocaryotic cell with unclamped septa (Giemsa); 2: Binucleate dicaryotic cell with clamp connections (Giemsa); 3: Acute-angled branching of dicaryotic hyphae (Giemsa); 4: Basidium in prophase I (Lu stain). 5: Hymenium (Lu stain); 6: Basidium in metaphase II (Lu stain); 7: Last stage of the second division of meiosis, the basidium contains four nuclei, sterigmata are apparent (Lu stain); 8: Basidiospore initiation at the apex of sterigmata (Lu stain); 9: Basidium showing four basidiospores (Congo red stain); 10: Uninucleate basidiospores with one binucleate basidospore (Giemsa); 11: Binucleate chlamydospore in formation (Giemsa).

Cycle de reproduction de *Dichomitus squalens*. 1: Cellule uninucléée, sans anse d'anastomose aux cloisons (coloration au Giemsa); 2: Cellule binucléée avec anses d'anastomoses (coloration au Giemsa); 3: Ramification à angle aigu d'une hyphe dicaryotique; 4: Baside en Prophase I de méiose (coloration de Lu); 5: Hyménium (coloration de Lu); 6: Baside en Métaphase II de méiose; 7: Fin de méiose II, la baside contient quatre noyaux, les stérigmates sont en place (coloration de Lu); 8: Différenciation de la basidiospore à l'apex du stérigmate (coloration de Lu); 9: La baside porte les quatre basidiospores (coloration au rouge Congo). 10: Basidiospores uninucléées (coloration au Giemsa); 11: Formation de chlamydospore binucléée (coloration au Giemsa).

Microscopic observation failed to reveal heterocaryons in incompatible pairings in which the clamps could not fuse with the subterminal cell of the hypha.

Microscopic examination verified the presence of two nuclei in all mycelia in pairings forming connection clamps (Fig. 2,3).

Up to 88 % of fresh basidiospores could germinate in liquid malt extract as shown by microscope examination (Tab. 4). The germination rate was only around 5% in the same medium with agar. Because of asynchronous germination and clumping of mycelia, initiation of germination in liquid medium followed by inclusion in a solid medium was not reliable for the generation of mono-basidiospore isolates.

Basidiospores could be stored in a dry state at 4°C or lyophilised with constant germination rate while storing in a liquid medium at low temperature resulted in a decrease of germination rate.

Table 4: Germination of basidiospores of *D. squalens* in different cultivation conditions.
Tableau 4: Germination de basidiospores de *D. squalens* suivant différentes méthodes.

Cultivation condition	Germination rate (24°C)	Incubation time
3% malt extract (liquid medium)		
fresh basidiospores	88.3 ± 1.8	3 days
lyophilized basidiospores[1]	72.6 ± 7.2	4 days
3% malt extract agar	2.9 ± 0.4	6 days

1. lyophilization as described in Materials and Methods; lyophilization in sucrose instead of inositol yielded only 8.2 germination rate.

DISCUSSION

The life cycle of *D. squalens* is of the haplodicaryophasic type with a tetrapolar interfertility system (Burnett, 1975; Fincham et al., 1979). Our results are in accord with previous report by Nobles et al. (1957) and in contradiction with Mounce (1929). In this system two loci designated A and B need to be present as different alleles for the morphogenetic sequence characterising a dicaryon to take place. In our study no hemicompatible pairings (B=, A≠ or B≠, A=) could be recognised. In several species including *Schizophyllum commune*, common B heterocaryons can be observed in which apical cells contain paired nuclei and at cell division a clamp cell is formed and the two nuclei undergo conjugate division. However because septum dissolution cannot take place, the clamp cell fails to fuse with the subapical cell and the daughter cell. Such hemicompatible pairings were not observed in our study and common B crossings were not identified.

The presence of a single nucleus in the vast majority of basidiospores and in all mono-basidiospore isolates establish that mono-basidiospore iso-

lates are homocaryons. Meiosis takes place according to the classical scheme described in other basidiomycetes with the formation of 4 mononucleate basidiospores per basidium (Kühner, 1977). No post-meiotic division was observed in this study, but the existence of a few dinucleate basidiospores could be explained either by a post-meiotic division or by mitosis of a nucleus after migration into the basidiospore (Slezec, 1980). Empty basidiospores could be explained by failure of a nucleus to migrate into the basidiospore or by an loss of the nucleus during staining.

Fruiting within an hymenium is not synchronous as in several *Pleurotus* species from Unbellifers (Slezec, 1986). This is in contrast to *Coprinus* species (Lu, 1967; Manachère & Bastouill-Descollonges, 1982).

Fruiting is best obtained in the malt extract agar method. Baxter & Manis (1939) and Nobles (1948) previously reported fruiting in malt extract agar cultures in *D. squalens*. The cultivation conditions were however not entirely defined. The requirement for light is established in this study in accordance with Baxter & Manis (1939). Fruiting of *D. squalens* on wood meal medium was reported by Badcock (1944) with no quantification of the result. No fruiting on wood blocks was reported according to Baxter & Manis (1939) and Badcock (1944). The results in this study establish that *D. squalens* is able to fruit on wood in controlled conditions with light. The malt agar method is however far more rapid and results in much more basidiospores than conventional fruiting methods using wood blocks or wood meal as a substrate. The malt agar method is simple and asepsis is better ensured than in cultures with wood. This method can be used without problems in genetic studies.

The present study establishes *D. squalens* as a white-rot fungus with a simple life cycle in which all events take place according to classical schemes. In *D. squalens* basidiospores are homocaryons and, in dicaryons, the cells contain constantly two nuclei. This allows genetic studies to be carried out and interpreted simply. This is in contrast with the well studied *Phanerochaete chrysosporium* in which the life cycle is not entirely understood and agreed on.

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